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14. ABSTRACT

Medulloblastoma is the most common malignant brain tumor in children. We have found that Aspm, a gene required for normal brain growth in early childhood, becomes co-opted during medulloblastoma formation, to support tumor growth. We have found that Aspm supports growth by reducing stress to genomic DNA when cells divide. We have further found that targeting Aspm can reduce medulloblastoma growth.

15. SUBJECT TERMS
medulloblastoma, microcephaly, brain progenitors

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Medulloblastoma, the most common malignant brain tumor in children, occurs primarily during the first decase of life. Early childhood is the period of most rapid brain growth. We hypothesized that the mechanisms that support developmental brain growth in early life can also play a key role in medulloblastoma formation. We therefore tested whether Aspm, a gene that when deleted causes reduced brain growth, is essential for medulloblastoma tumorigenesis. The performance of this investigation under the mentorship of Dr. Timothy Gershon, presented Dr. Garcia with an ideal opportunity to develop scientific independence.

In the first year of the project, Dr. Garcia made substantial progress toward her 3 Specific Aims.

Aim 1 was to determine the role of Aspm in neural progenitor function. Dr. Garcia analyzed mice with Aspm deletion at successive ages. Dr. Garcia determined that Aspm deletion in mice does limit brain growth, as it does in humans with hereditary microcephaly (see Fig 1,2 attached manuscript). Importantly, Dr. Garcia made the surprising discovery that Aspm deletion does not reduce growth by decreasing the rate of neural progenitor proliferation, but rather by increasing the frequency with which progenitors undergo cell death (see Fig. 3,4 attached manuscript). Moreover, Dr. Garcia identified increased replication stress as the cause of cell death in Aspm-deleted progenitors (see Fig. 4 sttached manuscript). These findings provide a new perspective on the relationship between Aspm and progenitor proliferation and informed the investigations for Aim 2.

Aim 2 was to determine the role of Aspm in medulloblastoma. Dr. Garcia developed expertise in microscopy and real-time PCR and used these techniques to demonstrate Aspm up-regulation in medulloblastomas that form spontaneously in genetically engineered mice (see Fig.1 attached manuscript). Dr Garcia then interbred these medulloblastoma-prone mice with the Aspm deleted mice that she had generated and found that Aspm loss reduced tumor growth (see Fig. 6. attached manuscript). As in brain progentiros, Dr. Garcia forund that Aspm deletion increased the replication stress of tumor cells.

Dr Garcia has worked with Dr. Gershon, here mentor, to generate a manuscript describing her findings from Aims 1 and 2 as described above. This manuscript has been submitted to a peer-reviewed journal and is currently under review.

Aim 3 was to test the effect of conditionally deleting Aspm in mouse medulloblastoma after tumors formed. Dr. Garcia generated tumor-prone mice with tamoxifen-inducible deletion of Aspm. This experiment required the use of the slowly tumorigenic SmoA1 line, rather than the more rapidly tumor-inducing SmoM2 line used in Aim 2. The resulting mice are currently under study and have not yet reached an age at which tumors are expected to form. After the point of tumor formation, mice will be injected with tamoxifen to induce Aspm deletion and the effects on tumor growth and animal survival will be examined.

RESEARCH AND TRAINING ACCOMPLISHMENTS:

- Demonstration that Aspm regulated by Shh (Fig. 1 attached manuscript)
- Demonstration of Aspm in medulloblastoma (Fig. 1 attached manuscript)

- Demonstration that Aspm is required for postnatal brain growth (Fig. 2 attached manuscript)
- Finding of preserved proliferation with Aspm deletion (Fig. 3 attached manuscript)
- Finding of increased apoptosis and DNA damage with Aspm loss (Fig. 4 attached manuscript)
- Finding that p53 deletion rescues phenotype of Aspm deletion (Fig. 5 attached manuscript)
- Finding that Aspm deletion limits medulloblastoma growth (Fig. 6 attached manuscript)

REPORTABLE OUTCOMES:

- Manuscripts: Aspm sustains cerebellar growth and medulloblastoma formation by mitigating replication stress (submitted)
- Development of animal models: Aspm knock out mice ($Aspm^{-/-}$), Aspm conditional knockout mice ($Aspm^{f/f}$), medulloblastoma-prone Aspm conditional mice ($Aspm^{f/f}$;hGfap-cre;SmoM2)
- Employment or research opportunities received based on experience/training supported by this training award: Dr. Garcia was offered a second postdoctoral fellowship position at the BioDonostia Institute, San Sebstian, in the laboratory of Dr. Ander Matheu.

CONCLUSION: In the first year of this 3 year grant, the PI made striking findings that validated the overall hypothesis that microcephaly genes may be ideal new targets for brain tumor therapies. Dr. Garcia identified Aspm as a microcephaly gene that is overexpressed in medulloblastoma and required for tumor growth. In the course of this work, Dr. Garcia gained key expertise that enabled her to find a research position in her native country, where she will continue her brain tumor studies.

Aspm prevents microcephaly and sustains medulloblastoma growth by mitigating replication stress and progenitor apoptosis.

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Summary

Microcephaly and medulloblastoma are disorders of neural development that result respectively from inadequate or excessive progenitor proliferation. Disruption of genetic mechanisms that regulate brain size may contribute to both microcephaly and tumorigenesis. We have identified Aspm, a gene known to be mutated in familial microcephaly, as a target of Shh signaling that promotes proliferation of cerebellar progenitors that are medulloblastoma cells of origin. Constitutive activation of Shh signaling in cerebellar progenitors by mutant Smo aberrantly prolonged Aspm expression, which further persisted in Smo-induced medulloblastomas. Genetic deletion of Aspm did not impair neural progenitor proliferation or self-renewal, but rather reduced brain growth by increasing replication stress and neural progenitor apoptosis. Deletion of Aspm in mice with Smo-induced medulloblastoma reduced tumor growth while increasing replication stress. Co-deletion of Aspm and p53 rescued the survival of neural progenitors and blocked the growth restriction imposed by Aspm deletion. Our data show that Aspm functions to mitigate replication stress during symmetric cell division, causing microcephaly through progenitor apoptosis when homozygously mutated, and sustaining medulloblastoma growth when co-opted in tumorigenesis.

Background

Symmetric cell division serves a vital role of amplifying diverse progenitor populations during brain development and both inadequate and excessive amplification have deleterious consequences. Primary microcephaly is a rare neurodevelopmental disorder that results from a failure to expand cell populations during brain formation; patients with primary microcephaly have brains in which neuronal diversity is relatively

preserved, but the overall size of the brain is markedly reduced (Woods et al., 2005). In contrast, medulloblastoma demonstrates the pathological effect of over-amplifying progenitor populations. The most common malignant brain tumors in children, medulloblastomas are growing masses of monomorphic cells resembling neural progenitors. In their divergent pathologies, microcephaly and medulloblastoma underscore the importance of regulating symmetric cell division to control brain growth.

Aspm has been implicated in pathological states of both inadequate and excessive growth. Mutations in ASPM cause familial microcephaly (Bond et al., 2002; Bond et al., 2003; Woods et al., 2005). Studies in mice show that Aspm is expressed by multi-potent neural stem cells at sites of cerebral neurogenesis (Bond et al., 2002; Marinaro et al., 2011) and that genetic deletion of Aspm reduces brain growth (Fish et al., 2006). ASPM has also been detected in cancers, including gliomas (Horvath et al., 2006; Bikeye et al., 2010), medulloblastomas (Vulcani-Freitas et al., 2011), hepatocellular carcinomas (Lin et al., 2008) and cancers of the ovary (Bruning-Richardson et al., 2011) and pancreas (Wang et al., 2013). Thus loss of Aspm function reduces growth, while aberrant expression of Aspm is associated with growth excess.

Various functions of Aspm have been identified. In Drosophila the orthologous gene, asp, maintains mitotic spindle orientation during both mitosis and meiosis (Zhonget al.2005; Kaindl et al. 2010). The mouse homolog Aspm is required during brain development to maintain mitotic spindle organization and positioning and acts as a microtubule-organizing center (Higgins et al. 2010; Kaindl et al. 2010). Recently, Aspm has been demonstrated to modulate progenitor proliferation and migration through an interaction with the Wnt developmental signaling pathway (Buchman et al., 2011). While the net effect of these diverse functions is to support both physiologic and malignant

proliferation, it is essential to determine how these functions converge to regulate cellular mechanisms of growth control.

The symmetrical divisions of cerebellar granule neuron progenitors (CGNPs) present an opportunity to study Aspm function in both brain growth and tumorigenesis. CGNPs proliferate in the external granule layer (EGL) of the cerebellum to generate the largest population of neurons in the brain (Espinosa and Luo, 2008). CGNPs are also proximal cells of origin for medulloblastoma (Schüller et al., 2008; Yang et al., 2008). Importantly specific developmental signaling molecules, including Shh and Wnt regulate both CGNP proliferation and medulloblastoma pathogenesis (Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000; Zurawel et al., 2000; Ellison et al., 2011; Hatten and Roussel, 2011; Roussel and Hatten, 2011). Here we have found that Aspm is induced by Shh in CGNPs, that Aspm positively regulates cerebellar neurogenesis by mitigating replication stress, and that this function becomes co-opted in medulloblastoma.

Material and Methods

Mice

Transgenic Aspm-EGFP reporter mice (Tg(Aspm-EGFP)lH113Gsat/Mmucd) were obtained from GENSAT (New York, NY, USA) (Gong S et al. 2010). Aspm-creER mice were generously shared by Luca Muzio, PhD, San Raffaele Scientific Institute, Milan, Italy and have been previously described (Marinaro et al., 2011).$Aspm^{SA/SA}$ mice were generated from Aspm-targeted ES cells ($Aspm^{Gt(AA0137)Wtsi}$; KOMP Repository; Davis, CA, USA). $Aspm^{SA/SA}$ mice were crossed with FLPeR mice (strain: 3946; Jackson Laboratories, Bar Harbor, ME, USA) to generate Aspm floxed ($Aspm^{f/+}$) mice. Math-1 cre mice were generously shared by David Rowitch, MD, PhD, UCSF and Robert Wechsler-Reya, PhD,

Sanford-Burnham Medical Research Institute, La Jolla, CA and have been previously described (Matei et al., 2005). hGFAP-cre mice were generously provided by Eva Anton,PhD, University of North Carolina, Chapel Hill, NC, USA; these mice express cre during brain development in stem cells that give rise to diverse progeny, including the entire cerebellum (Zhuo et al., 2001). SmoM2 mice (strain:5130) and TdTomato reporter mice (strain:7914) were obtained from Jackson Laboratories, Bar Harbor, ME, USA. Medulloblastoma-prone NeuroD2:SmoA1 mice were kindly provided by James Olson, MD, PhD, Fred Hutchinson Cancer Research Center, Seattle, WA, USA (Hallahan et al., 2004). Conditional 53 floxed ($p53^{f/f}$) mice (strain : 01XC2) were provided by the NCI, Frederick, MD. All the mice were crossed thorugh at least 4 generations into a C57BL/6 genetic background. Mice of either sex were used in experiments. All animal handling and protocols were carried out in accordance with established practices as described in the National Institutes of Health Guide for Care and Use of Laboratory Animals and as approved by the Animal Care and Use Committee of the University of North Carolina (IACUC# 10-126).

CGNP culture

CGNPs were isolated and explanted as previously described (Kenney et al., 2003). Cells were maintained in 0.5μg/ml of Shh (R&D Systems Minneapolis, MN, USA) or vehicle 0.5%BSA-PBS1x) as indicated.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was isolated from cerebella, and from explanted CGNP using the RNeasy kit (Qiagen, Valencia, CA ,US), following manufacturer protocol. Oligo dT-proimed

cDNA was synthesized from 1µg total RNA using the Superscript III kit (Invitrogen/Life technologies, Grand Island, NY, US).), per manufacturer protocol. Gene expression was quantified on an ABI PRISM 7500 Sequence Detection System, using the $\Delta\Delta C_T$ relative quantification method. All experiments included no template controls and were performed in triplicate and repeated twice independently. Transcript levels were normalized to GAPDH reference gene. The primers used for the amplification of Aspm have been previously published and validated and were GCTTCATCACCTGCTCACCTAC and GTAGATACCGCTCCGCTTTCAG (Wu et al., 2008). Additional primer pairs were Cyclin D2 GCGTGCAGAAGGACATCCA and CACTTTTGTTCCTCACAGACCTCTAG, GAPDH TGTGTCCGTCGTGGATCTGA and CCTGCTTCACCACCTTCTTGA.

In vivo proliferation analysis

Aspm-GFP mouse pups were injected at P7 IP with 50 µl HBSS containing EdU (250 µM, Invitrogen/Life technologies, Grand Island, NY, US). After 24 hours, brains were dissected and incubated in 4% formaldehyde in PBS for 24 hours at 4ºC, then processed for histology. EdU was detected following manufacturer's protocol.

Histology, immunochemistry and cell quantification.

Mouse brains were processed and immunohistochemistry was performed as previously described (Garcia et al. 2012; Gershon et al. 2009), using the following primary antibodies:cC3 (Cell Signaling, Boston, MA, USA, cat#9661), PCNA (Cell Signaling, Boston, MA, USA), , PH3 (Cell Signaling, Danvers, MA, USA, cat#9706), and γH2AX (Cell Signaling, Boston, MA, USA, cat#9718). Stained slides were imaged with an Aperio Scanscope digitizing microscope. To quantify the number of positive cells in the

cerebellum, slides were analyzed using Aperio Software (Aperio, Vista, CA, USA) for DAB stained tissue or Tissue Studio (Definiens, München, Germany), for fluorescence stained samples. Statistical comparisons were made using 2-sided Student's t test.

Western blot analysis

Proteins of whole cerebella from $Aspm^{+/+}$ and $Aspm^{-/-}$ mice were extracted and processed as previously described (Garcia et al. 2012). Immunologic analysis was performed on a SNAP ID device (Millipore, Billerica, MA, USA) using primary antibodies to γH2AX and β-Actin (Cell Signaling, Boston, MA, USA, cat#4970).

Results

Aspm is induced by Shh and up-regulated during cerebellar neurogenesis

To visualize the cellular pattern of Aspm expression in the postnatal brain, we examined Aspm-GFP reporter mice generated by the Gensat project. To identify proliferating cells in these mice, we injected pups with EdU 24 hours before harvesting them. In the brains of mice at postnatal day 7 (P7), we noted that sites of GFP expression clorresponded with the sites postnatal neurogenesis, including the EGL (Fig 1A). In the cerebellum, GFP expression followed the temporal pattern of CGNP proliferation, waning by P16 (Fig. 1B). The relationship between Aspm expression and CGNP proliferation was further demonstrated by real-time, quantitative PCR (qPCR), as Aspm expression mirrored the expression of Cyclin D2, a previously identified marker of CGNP proliferation (Kenney and Rowitch, 2000). The expression of both Cyclin D2 and Aspm was relatively high at peak neurogenesis (P7), and waned by the neurogenic period (P16; Fig. 1C). Importantly, isolated CGNPs when maintained in a proliferative state by exposure to exogenous Shh,

expressed significantly more Aspm mRNA compared to Shh-deprived CGNPs (Fig. 1C), and CGNPs isolated from Aspm-GFP mice up-regulated GFP when maintained in Shh (Fig. 1D).

To determine whether Shh-pathway activation induces Aspm *in vivo*, we crossed Aspm-GFP reporter mice with the transgenic, medulloblastoma-prone ND2:SmoA1 mouse line. ND2:SmoA1 mice harbor a constitutively active allele of Smoothened, expressed under control of the NeuroD2 promoter. The Shh signaling pathway is activated in a cell autonomous manner in CGNPs of ND2:SmoA1 mice, which prolongs the period of CGNP proliferation and predisposes these mice to medulloblastoma (Hallahan et al., 2004). In Aspm-GFP;ND2:SmoA1 mice, CGNPs proliferated beyond the typical period of 15 days, and continued to express the Aspm-GFP reporter (Fig. 1E). As spontaneous medulloblastomas developed in Aspm-GFP;ND2:SmoA1 mice, the Aspm-GFP reporter was consistently expressed throughout the resulting tumors (n>15; Fig. 1F). Activation of the Shh signaling pathway was thus sufficient to drive Aspm expression *in vivo*, and Aspm expression was a consistent feature of Shh-driven medulloblastoma.

To examine the fate of postnatal Aspm expressing progenitors, we bred Aspm-creER;tdTomato mice, which express a tamoxifen-inducible, fluorescent reporter in Aspm expressing cells. We injected tamoxifen at P4 and P7, and then harvested the mice at P11. We detected red fluorescence, indicating Aspm+ lineage, in CGNPs of the EGL, in progenitors of the hippocampus and RMS, in CGNs of the IGL, and in small CD31+ blood vessels throughout all regions of the brain (Fig. 1G,H). Thus in the post-natal brain, the set of cells that derive from Aspm-expressing progenitors includes neural progenitors, differentiated neurons, and capillary endothelial cells.

Aspm in CGNPs is required for cerebellar growth

To determine if Aspm function is critical to CGNP proliferation, we examined the effect of Aspm-deficiency on cerebellar growth. We generated Aspm-deficient mice from $Aspm^{Gt(AA0137)Wtsi}$ Knock-Out First ES cells (KOMP, USA). ES cells were injected into blastocysts to generate chimeras. Chimeras were then crossed with albino Bl6 mice, and the resulting black mice were genotyped by PCR to identify mice heterozygous for insertion of a splice-acceptor cassette between exons x and x of Aspm. The selected $Aspm^{SA/+}$ mice were then crossed back into Bl6 mice and then intercrossed to generate $Aspm^{SA/SA}$, $Aspm^{SA/+}$, and $Aspm^{+/+}$ littermates.

We verified Aspm deficiency using qPCR, which demonstrated that Aspm mRNA were significantly less abundant in $Aspm^{SA/SA}$ mice, compared with $Aspm^{SA/+}$, and $Aspm^{+/+}$ littermates (Fig 2A). The EGL of $Aspm^{SA/SA}$ mice was notably thinner at P13 compared to $Aspm^{+/+}$ littermates, consistent with premature waning of the CGNP population (Fig 2B). Compared to $Aspm^{SA/+}$ or $Aspm^{+/+}$ littermates, $Aspm^{SA/SA}$ mice had smaller brains (Fig. 2C) with smaller cerebella (Fig 2D). Thus $Aspm^{SA/SA}$ mice phenocopied previously reported Aspm-deleted mice (Pulvers et al., 2010), and Aspm-deficiency reduced the size of the cerebellum along with the rest of the brain.

To determine whether deletion of Aspm impaired cerebellar growth through a cell autonomous effect on CGNPs, we used conditional deletion to ablate Aspm in the Math1 lineage. We generated mice with conditional deletion of Aspm and crossed them with Math1-cre mice (Helms et al., 2000; Machold and Fishell, 2005; Matei et al., 2005). Aspm mRNA abundance was reduced in cerebella of Math1-cre;$Aspm^{fl/fl}$ mice, compared to Math1-cre;$Aspm^{f/+}$ littermates (Fig 2E). While overall brain weight was relatively preserved

in Aspm$^{fl/fl}$;Math1-cre mice (Fig. 2F), cerebellum weight was significantly lower compared to littermate controls (Fig 2G). Conditional deletion of Aspm in the Math1 lineage thus confirmed that Aspm maintains cerebellar growth by acting on CGNPs to support the expansion of their population.

Proliferation is not impaired Aspm-deficient CGNPs

Population growth integrates proliferation, differentiation and apoptosis (Haydar et al., 1999; Woods et al., 2005; Poulton et al., 2011). The postnatal proliferation of CGNPs presents an ideal opportunity to examine proliferation dynamics in Aspm-dependent progenitors. To investigate the effect of Aspm deficiency on CGNP proliferation, we examined cerebella of Aspm$^{SA/SA}$ and Aspm$^{+/+}$ mice at different time-points during postnatal neurogenesis (P7, P11, P13, and P15), representing the typical peak and waning periods of CGNP proliferation. At P7 and P11, mitotic figures, highlighted by IHC for PH3 were readily detected in the EGL of Aspm$^{SA/SA}$ mice (Fig. 3A). Quantification of PH3+ cells in the EGL of Aspm$^{SA/SA}$ and Aspm$^{+/+}$ mice demonstrated no statistically significant difference in mitotic rate (Fig. 3B). With the waning of proliferation at P13 and P15, mitoses were markedly less frequent in both genotypes, complicating quantification of PH3+ cells. We therefore compared the number of cells expressing proliferation marker PCNA, which is expressed more broadly during the cell cycle (Fig. 3C). At each time point, we did not detect statistically significant difference in the proportion of PCNA+ CGNPs in Aspm$^{SA/SA}$ and Aspm$^{+/+}$ mice (Fig. 3D).

To determine if Aspm deficiency altered the mitogenic effect of Shh, we isolated CGNPs from Math1-cre;Aspm$^{f/f}$ mice and from Aspm$^{f/f}$ littermates without the Math1-cre, and then cultured these CGNPs in 4 replicate wells per genotype, in the presence of Shh

for 48 hours. Quantitative IHC for PH3 demonstrated that Aspm-deficient CGNPs from Math1-cre;Aspm$^{f/f}$ mice maintained proliferation in the presence of Shh as effectively as CGNPs with intact Aspm (Fig. 3E). Thus Aspm deficiency did not detectably reduce either proliferation in vivo, or proliferative response to Shh in vitro.

Aspm deficiency increases progenitor apoptosis and replication stress.

While Aspm deficiency did not impair neural progenitor proliferation, apoptosis of neural progenitors was significantly increased. We quantified apoptosis using IHC for cC3, and determining the fraction of cells in the EGL that were cC3+. We compared Aspm$^{SA/SA}$ and Aspm$^{+/+}$ genotypes at P7 and P11. We have previously shown that cC3+ cells are rarely detected in the EGL wild-type mice (Garcia et al., 2012). In contrast, apoptotic cells were significantly more frequent in the EGL of Aspm$^{SA/SA}$ mice (Fig. 4A-C). Increased apoptosis with Aspm deficiency was proportional to proliferation, highest at P7 and lower at P11. Conditional deletion of Aspm in CGNPs also increased apoptosis, as cC3+ cells were more frequent in the EGL of P7 Math1-cre;Aspm$^{f/f}$ mice compared to Math1-cre;Aspm$^{f/+}$ controls (0.87% vs 0.23%, p=0.006). No significant difference was found between Math1-cre;Aspm$^{f/+}$ and no cre littermates (data not shown). These data show that Aspm deficiency increased CGNP apoptosis in a cell autonomous manner that was linked to proliferation.

CGNPs are highly sensitive to apoptosis in response to genotoxic stress (Chong et al., 2000; Lee et al., 2012). The known role of Aspm in mitotic spindle dynamics (Zhong et al., 2005; Fish et al., 2006; Higgins et al., 2010) suggested that the increased cell death with Aspm deficiency might be caused by increased chromosomal damage during mitosis. Accordingly we used quantitative IHC for phosphorylated Histone H2AX (γH2AX) in order

to determine if DNA damage was more frequent in Aspm-deficient CGNPs. We compared $Aspm^{SA/SA}$ and $Aspm^{+/+}$ genotypes at P7 and P11. We found scattered γH2AX+ cells in the EGL in all mice (Fig. 4D,E), demonstrating that typical CGNP proliferation causes detectable replication stress. $Aspm^{SA/SA}$ mice had significantly more γH2AX+ CGNPs in the EGL at P7, compared to $Aspm^{+/+}$ mice (Fig. 4E,F). In contrast, at P11, when proliferation was waning, the effect of Aspm deficiency was markedly reduced. Thus Aspm deficiency increased the rate of proliferation-related DNA damage during postnatal neurogenesis, identifying increased replication stress as a potential mechanism inducing progenitor apoptosis with Aspm loss of function.

P53 deletion rescues Aspm-induced microcephaly

Replication stress can trigger neural progenitor apoptosis through p53 dependent mechanisms (Vousden and Lu, 2002; Fridman and Lowe, 2003; Lee and McKinnon, 2007). To determine if increased replication stress was the cause of CGNP apoptosis and growth restriction in Aspm-deficient mice, we investigated whether deletion of p53 rescued Aspm-deficient CGNPs. We crossed Math1-cre;$Aspm^{f/f}$ and $p53^{f/f}$ mouse lines and selected Math1-cre;$Aspm^{f/f}$;$p53^{+/+}$ and Math1-cre;$Aspm^{f/f}$;$p53^{f/f}$ progeny for comparison. We harvested mice at P7 and compared the rates of apoptosis, DNA damage, and proliferation, by determining the proportion of cells in the EGL that were, respectively cC3+, γH2AX+ and PH3+. We found that CGNP apoptosis was significantly reduced in Math1-cre;$Aspm^{f/f}$;$p53^{f/f}$ mice, compared to Math1-cre;$Aspm^{f/f}$ mice with wild type p53 (Fig. 5A). In contrast, we did not detect significant changes in DNA damage or proliferation rate (Fig. 5B,C). We then raised Math1-cre;$Aspm^{f/f}$;$p53^{f/f}$ mice to P30 and compared cerebellum weight to that of Math1-cre;$Aspm^{f/f}$ mice with wild type p53. We found that cerebellum

weight was significantly increased by deletion of p53 (Fig. 5D). Taken together, these data support a model in which Aspm supports brain growth by reducing replication stress during neural progenitor proliferation.

Aspm deficiency slows the growth of medulloblastoma

Because of the consistent finding of Aspm up-regulation in medulloblastoma, both ND2:SmoA1 mice (Fig.1F) and patient-derived samples (Vulcani-Freitas et al., 2011), we examined if Aspm function is critical to tumor growth. To test this possibility, we bred Aspm conditional deletion into the rapidly tumorigenic medulloblastoma model, hGFAP-cre;SmoM2. Transgenic SmoM2 mice express a consititutively active, cre-inducible allele of the Shh receptor component Smo (Mao et al., 2006). Transgenic hGFAP-cre mice undergo recombination of floxed genes in stem cells that give rise to the neurons and glia of the cerebrum and cerebellum (Zhuo et al., 2001). Mice with the genotype hGFAP-cre;SmoM2 develop medulloblastoma with 100% incidence and die of tumor progression by P20 (Schüller et al., 2008). We investigated the effect of Aspm deletion on tumor growth by comparing hGFAP-cre;SmoM2 mice with $Aspm^{fl/fl}$ or $Aspm^{fl/+}$ genotypes.

We found that Aspm-deficient medulloblastomas grew significantly more slowly than tumors with an intact Aspm allele. The rate of tumor formation was 100% in both genotypes. Tumors could be differentiated from normal brain at P3, and at this early point, tumor size appeared to be similar in $Aspm^{fl/fl}$ or $Aspm^{fl/+}$ mice (Fig. 6A,C). Between P3 and P13, however, $Aspm^{fl/fl}$ tumors grew more slowly than $Aspm^{fl/+}$ tumors, such that by P13 Aspm-deficient tumors were significantly smaller (Fig. 6B,D). Comparison of the ratios of tumor to non-tumor cerebellum at P13 demonstrated significant difference in the rate of tumor growth (Fig. 6E). Within tumors of both genotypes, γH2AX+ cells were too numerous

and densely packed to be quantified by IHC. Using Western blot, however, we consistently found significantly increased γH2AX+ in Aspm-deleted tumors at P3 (Fig. 6F). By P13, however, γH2AX+ abundance was equivalent (data not shown), suggesting that tumors had developed a mechanism to compensate for Aspm deletion. Consistent with the evolution of resistance within tumors, hGFAP-cre;SmoM2;Aspm$^{fl/fl}$ mice demonstrated progressive neurologic abnormality, could not be weaned, and did not survive longer than hGFAP-cre;SmoM2;Aspmf/+ mice with more rapidly expanding tumors. Thus, while we did not detect a survival benefit, Aspm deletion impacted malignant growth in a manner similar to physiologic development, increasing replication stress during medulloblastoma tumorigenesis and restricting tumor growth.

Discussion

In this investigation, we have shown that Aspm reduces replication stress during both physiologic and malignant proliferation, and that Aspm deletion causes microcephaly by increasing progenitor cell death. We found that Aspm is induced in CGNPs by Shh signaling and remains up-regulated in in Shh-induced medulloblastoma. Aspm disruption reduced brain growth globally and conditional deletion of Aspm in CGNPs similarly restricted the growth of the cerebellum. Importantly, Aspm deletion did not reduce CGNP proliferation, but rather increased CGNP apoptosis. The increased cell death associated with Aspm deletion was accompanied by increased DNA damage in proliferating CGNPs. Aspm deletion also increased DNA damage early in the process of medulloblastoma pathogenesis and coincided with restricted tumor growth between P3 and P13. Apoptosis of Aspm-deficient CGNPs was prevented by co-deletion of p53, consistent with genotoxic injury as the cause of cell death. Moreover, the restoration of cerebellar growth in mice

with co-deletion of Aspm and p53 demonstrates that preventing apoptosis can rescue Aspm-dependent growth restriction.

Human and mouse studies have shown that microcephaly can result from either loss of progenitor self-renewal, as in premature differentiation of Nde1-deleted progenitors (Pawlisz et al., 2008), or from progenitor apoptosis, as seen in Cdk5rap2 mutation (Pawlisz et al., 2008; Lizarraga et al., 2010). These two mechanisms may also operate simultaneously, for example centrosome amplification (Marthiens et al., 2013) and Magoh mutation (Silver et al., 2010) cause microcephaly by inducing both apoptosis and loss of self-renewal in neural progenitors. In all of these examples, the phenotype produced in mice is severe brain malformation. In both our genetic model and an independent genetrap model, however, Aspm deletion produced a phenotype in mice that is much less severe than the orthologous phenotype in humans (Pulvers et al., 2010). This variance from the typical pattern of microcephaly genes suggests that the mechanism of microcephaly in Aspm deletion may be critically different.

Our data indicate that loss of Aspm function restricts brain growth by modestly increasing the probability of progenitor cell death during symmetric cell division. Increased DNA damage mediates this increased risk of apoptosis. Specific genes, for example, Atr, have been shown to be required for symmetric cell division without lethal replication stress (Lee et al., 2012). Unlike Atr, however, which causes severe phenotype when deleted, Aspm is not required for most symmetric divisions to produce viable progeny. We have previously shown that even a very low rate of apoptosis, detectable only by comparing to apoptosis-deficient, Bax-deleted mice, exerts a significant impact on cerebellar growth (Garcia et al., 2012). We propose that Aspm loss imposes a cost to the developing brain through increased apoptosis that is proportional to the requirement of the organism for

brain growth; thus Aspm deletion is more eloquent in humans, in which brain size is larger and the period of brain growth is more prolonged.

The marked restriction of tumor growth in Aspm-deleted medulloblastoma is consistent with our model in which the effect of Aspm loss is proportional to the extent of proliferation. In both cerebellar development and medulloblastoma formation, Aspm deletion increased replication stress. In tumors, however, where proliferation is more extensive than in normal tissues, DNA damage and growth restriction with Aspm loss were most pronounced. Importantly, previous investigators have demonstrated that Aspm knockdown impairs DNA repair after ionizing radiation in vitro (Kato et al., 2011). Our findings that medulloblastomas require Aspm for typical growth suggest that Aspm could be therapeutically targeted to increase the efficacy of radiation and chemotherapy for medulloblastoma. Primary mouse medulloblastoma with Aspm deletion provides an ideal model to test this possibility in preclinical studies.

Figure Legends

Figure 1. Aspm is up-regulated by Shh in CGNPs and medulloblastoma. **A)** Sagittal section of an Aspm-GFP reporter mouse brain at P7 shows GFP expression corresponds with zones of postnatal neurogenesis labeled by EdU injection at P6. Bar = 1mm. **B)** GFP expression in Aspm-GFP reporter mice wanes by P16. White arrows indicate the EGL. Bar = 500μm. **C)** real-time RT-PCR demonstrates Aspm mRNA is markedly less abundant in developing cerebellum P16 compared to P7, and markedly down-regulated when isolated CGNPs are maintained in vitro without Shh. **D)** Isolated CGNPs from Aspm-GFP reporter mice express GFP only when cultured with Shh. Bar = 100μm **E)** Sagittal section through the cerebellum of a ND2:SmoA1;Aspm-GFP mouse shows continued expression of GFP

at P16, in contrast to **(B)**. White arrows indicate the persistent EGL resulting from Smo activation. Bar = 500μm **F)** Medulloblastomas in ND2:SmoA1;Aspm-GFP mice express GFP specifically in tumor cells. Bar = 50μm **G,H)** Section of cerebellum of an Aspm-creER;tdTomato reporter mouse injected with Tamoxifen at P4 and P7 then harvested at P11, demonstrates progenitors in the EGL, neurons in the IGL and endothelial cells (white arrows) all descend from Aspm-expressing cells. Bar = 100μm in (G) and 50 μm in (H).

Figure 2. Reduced brain growth and postnatal cerebellar neurogenesis in $Aspm^{SA/SA}$ and Math1-cre;$Aspm^{f/f}$ mice. **A)** Aspm mRNA is significantly reduced in $Aspm^{SA/SA}$ mice (n=3). **B)** Representative sagittal sections of cerebella show that EGL thickness was reduced in $Aspm^{SA/SA}$ mice compared to Aspm wild-type littermates (n=3). Bar = 500μm. **C,D)** Brain weight and weight of cerebella were significantly decreased in $Aspm^{SA/SA}$ mice compared to both $Aspm^{SA/+}$ and Aspm wild-type littermates. **E)** Aspm mRNA is significantly reduced in Math1-cre;$Aspm^{f/f}$ mice compared to $Aspm^{f/+}$;Math1-cre littermates (n=3). **F,G)** Cerebellum weight was significantly decreased in Math1-cre;$Aspm^{f/f}$ mice compared to littermate controls (n=6), while the overall weight of the brain did not change in a statistically significantly manner (n=4).

Figure 3. Proliferation is not reduced by Aspm disruption. **A,C)** Representative images of mouse cerebella in sagittal section at P7 stained for PH3 **(A)** or P15 stained for PCNA **(C)**. Genotype is indicated. **B,D)** $Aspm^{SA/SA}$ genotype did not cause a statistically significant change in the proportion of CGNPs in the EGL that were PH3+ at P7 or P11, nor in the proportion of CGNPs that were PCNA+ at P13 and P15. **E)** The proliferative response of Math1-cre;$Aspm^{f/f}$ CGNPs to Shh in vitro was not significantly different from the response of CGNPs from littermate controls. Bar = 250μm in (G) and 100 μm in (H).

Figure 4. Aspm disruption increased CGNP apoptosis and replication stress. **A,B)** Representative images of cerebella in sagittal section from (A) $Aspm^{+/+}$ and (B) $Aspm^{SA/SA}$ mice at P7 stained for cC3 (black arrows). **C)** Quantification of cC3+ cells in the EGL of $Aspm^{SA/SA}$ and wild-type littermates demonstrates statistically significant increase in cell death with Aspm deficiency at P7 and P11. **D,E)** Representative images of cerebella from (D) $Aspm^{+/+}$ and (E) $Aspm^{SA/SA}$ mice at P7 stained for γH2AX (red arrows). **F**) Quantification of γH2AX+ demonstrates statistically significant increase in cells with DNA damage at P7.

Figure 5. Co-deletion of p53 reduced the pro-apoptotic affect of Aspm deletion. **A-C)** Quantification of cC3+ (A), γH2AX (B) and PH3 (C) CGNPs in the EGL of P7 mice, comparing Math1-cre;Aspmf/f and Math1-cre;$Aspm^{f/f;}p53^{f/f}$ genotypes (n=4). The rate cell death in Aspm deficient CGNPs was reduced by p53 deletion, while the rates of CGNPs with DNA damage (γH2AX+) or undergoing mitosis (PH3+) were not significantly changed. **D)** Cerebellum growth was restored in P30 Math1-cre;$Aspm^{f/f}$;$p53^{f/f}$ mice compared to Math1-cre;$Aspm^{f/f}$;$p53^{+/+}$ littermates (n=4).

Figure 6. Conditional deletion of Aspm in medulloblastoma slows tumor growth A,B) Tumors in Math1-cre;$Aspm^{f/+}$;SmoM2 mice were detectable at P3 and grew rapidly by P13. C,D) Aspm-deficient tumors in Math1-cre;$Aspm^{f/+}$;SmoM2 mice were similarly detectable at P3 but grew more slowly from P3 to P13 (n=3). E) Proportion of cross sectional area of cerebellum composed of tumor at P3 and P13. F) Western blot for γH2AX shows consistently increased levels of DNA damage in Aspm-deficient tumors in Math1-cre;$Aspm^{f/+}$;SmoM2 mice.

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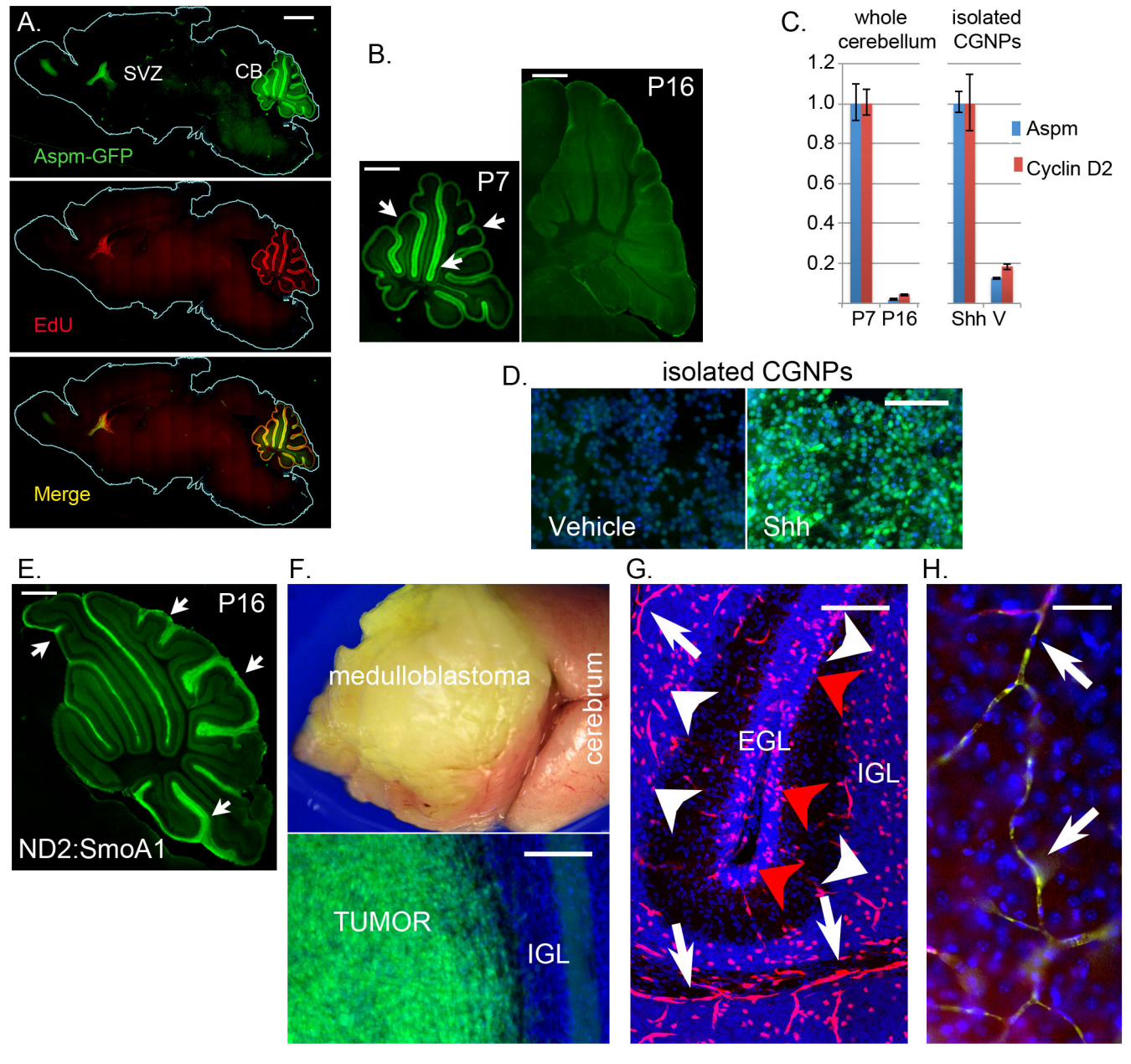
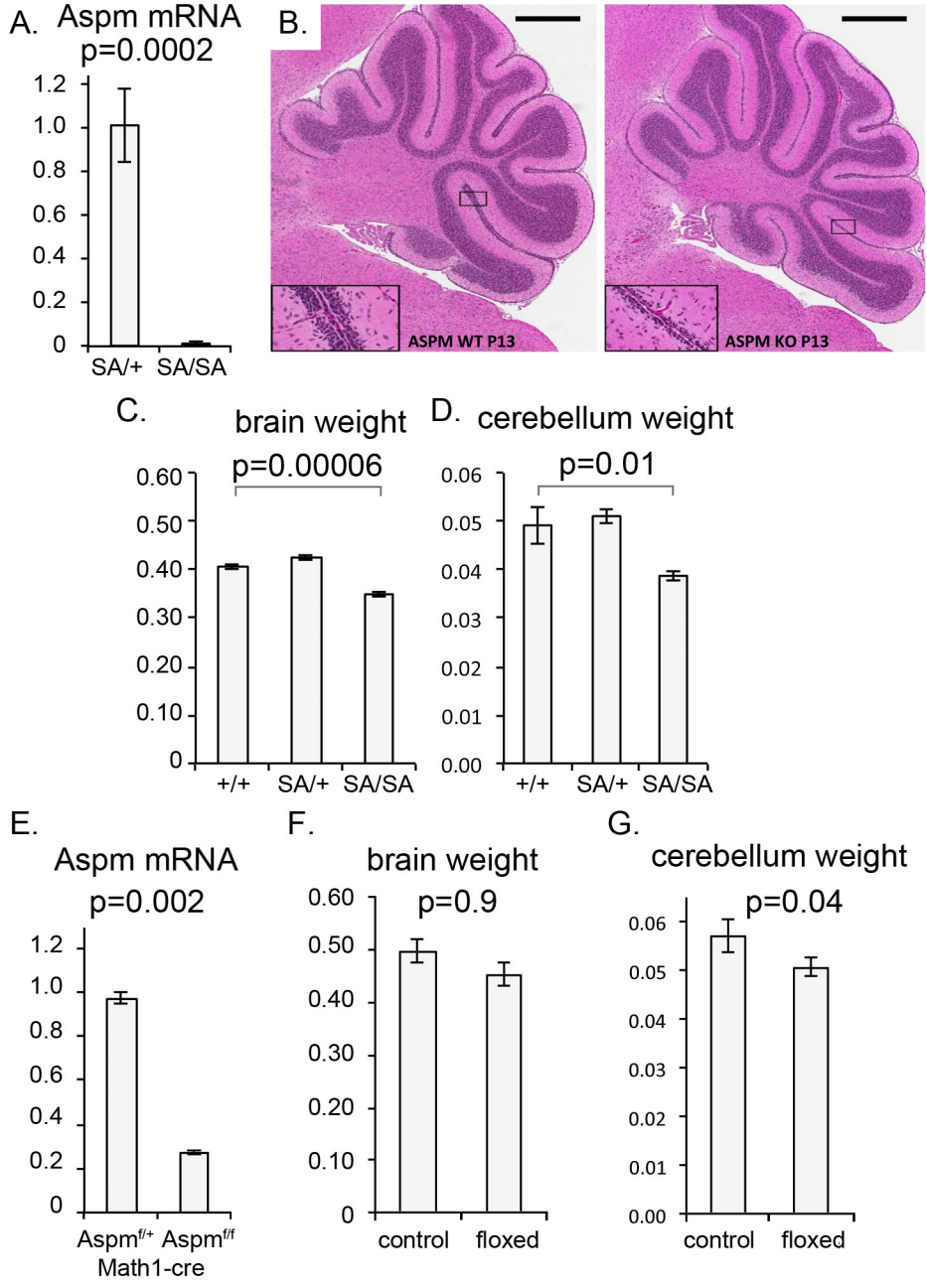

A.
SVZ
CB
Aspm-GFP
EdU
Merge
B.
P7
P16
C.
whole cerebellum
isolated CGNPs
1.2
1.0
0.8
0.6
0.4
0.2
Aspm
Cyclin D2
P7 P16
Shh V
D.
isolated CGNPs
Vehicle
Shh
E.
P16
ND2:SmoA1
F.
medulloblastoma
cerebrum
TUMOR
IGL
G.
EGL
IGL
H.


A.
Aspm mRNA
p=0.0002
1.2
1.0
0.8
0.6
0.4
0.2
0
SA/+
SA/SA
B.
ASPM WT P13
ASPM KO P13
C.
brain weight
p=0.00006
0.60
0.50
0.40
0.30
0.20
0.10
0
+/+
SA/+
SA/SA
D.
cerebellum weight
p=0.01
0.06
0.05
0.04
0.03
0.02
0.01
0.00
+/+
SA/+
SA/SA
E.
Aspm mRNA
p=0.002
1.2
1.0
0.8
0.6
0.4
0.2
0
Aspmf/+
Aspmf/f
Math1-cre
F.
brain weight
p=0.9
0.60
0.50
0.40
0.30
0.20
0.10
0
control
floxed
G.
cerebellum weight
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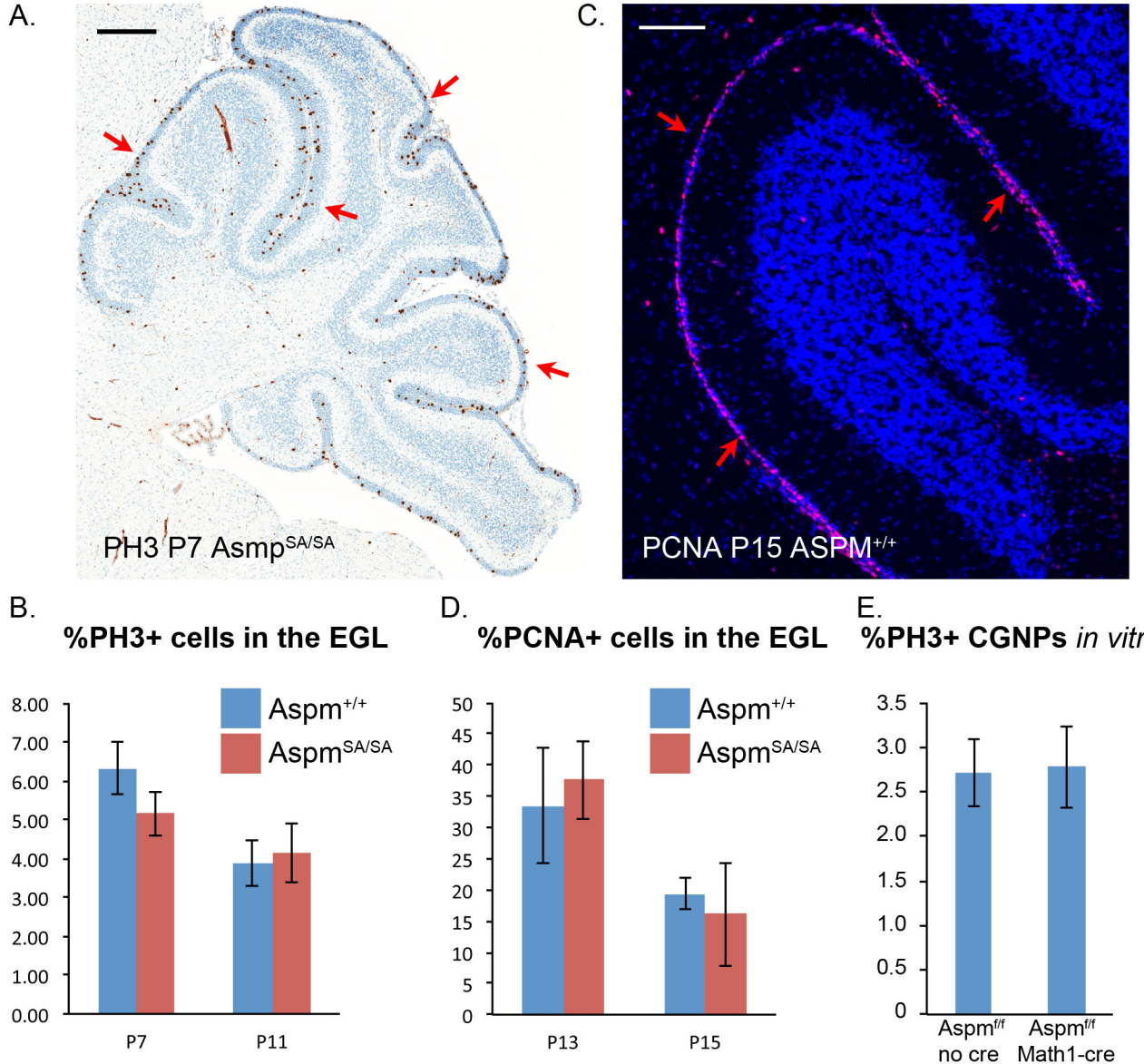
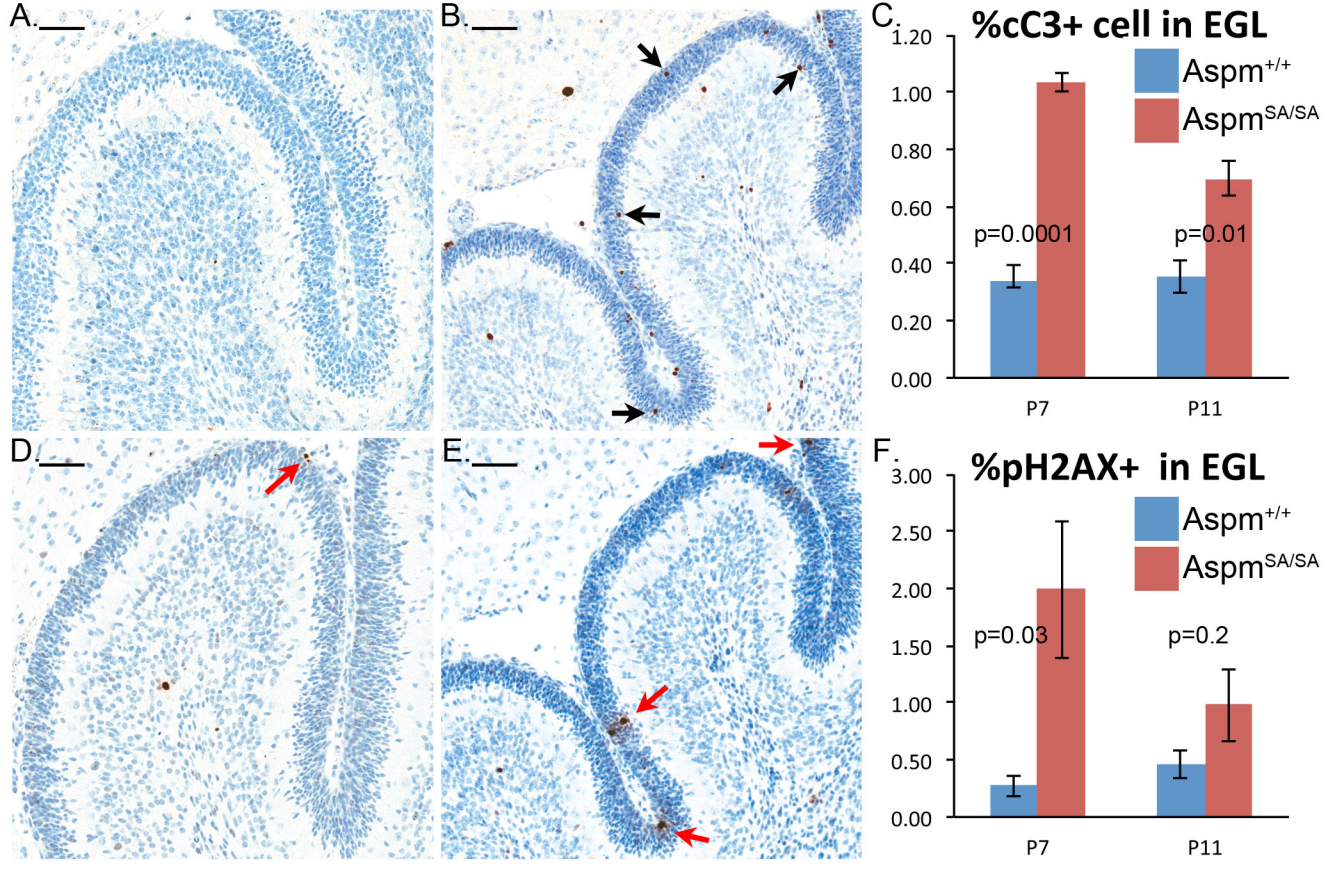
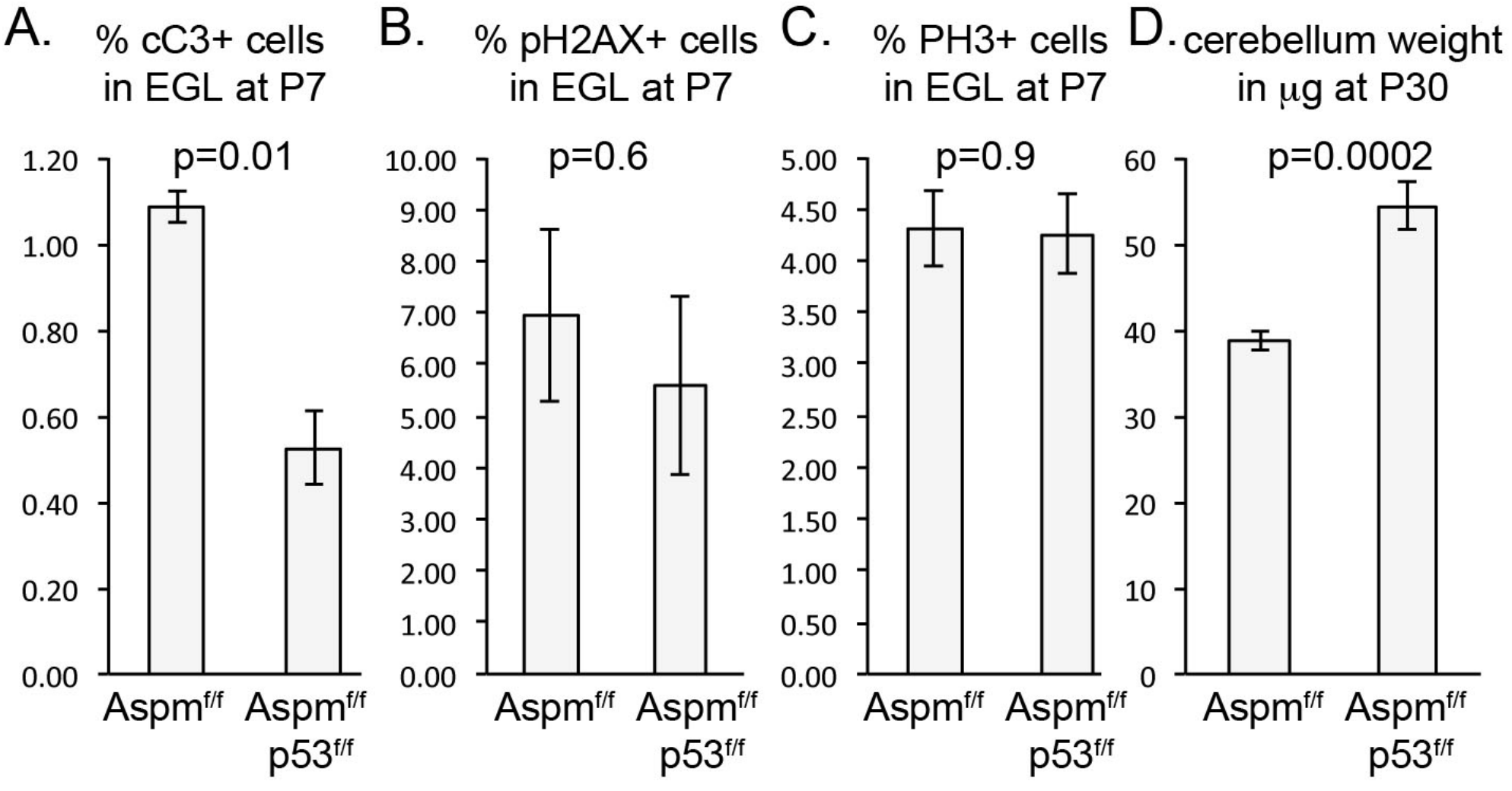
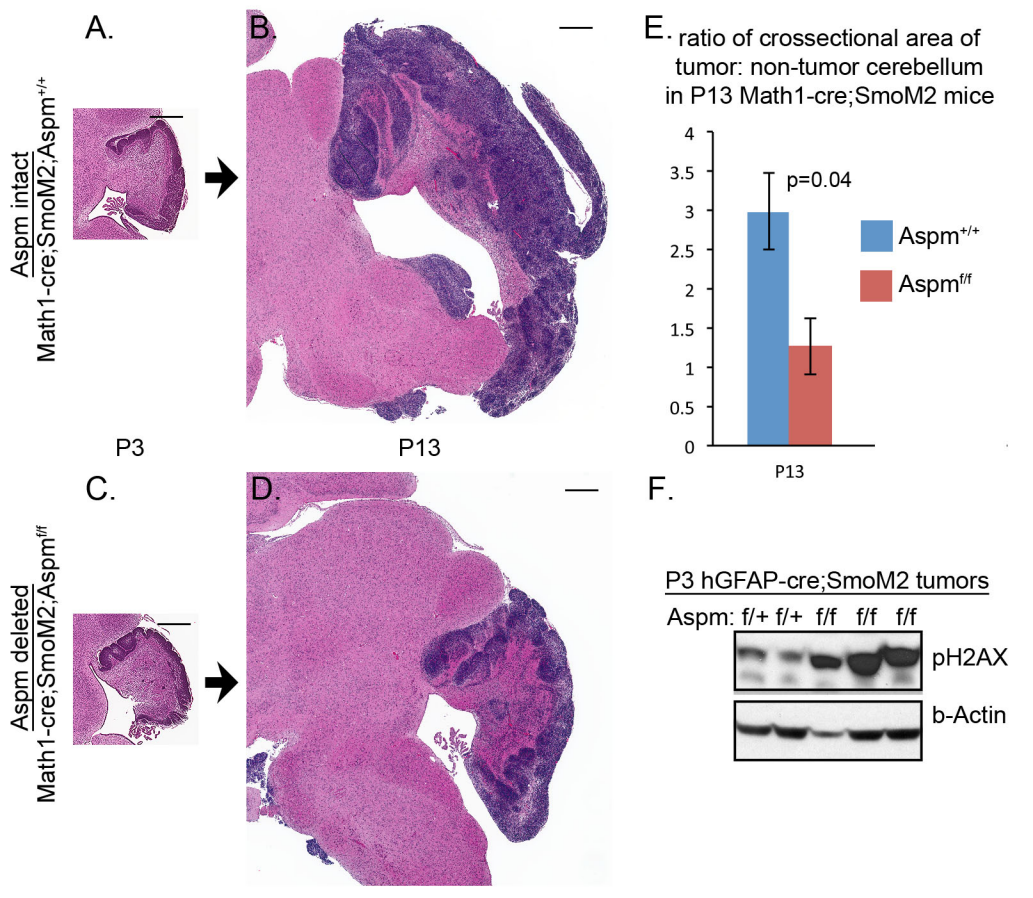
0.06
0.05
0.04
0.03
0.02
0.01
0.00
control
floxed


A.
PH3 P7 Asmp$^{SA/SA}$
C.
PCNA P15 ASPM$^{+/+}$
B.
%PH3+ cells in the EGL
Aspm$^{+/+}$
Aspm$^{SA/SA}$
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
P7
P11
D.
%PCNA+ cells in the EGL
Aspm$^{+/+}$
Aspm$^{SA/SA}$
50
45
40
35
30
25
20
15
10
5
0
P13
P15
E.
%PH3+ CGNPs *in vitro*
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Aspm$^{f/f}$ no cre
Aspm$^{f/f}$ Math1-cre


A.
B.
C.
%cC3+ cell in EGL
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Aspm+/+
AspmSA/SA
p=0.0001
p=0.01
P7
P11
D.
E.
F.
%pH2AX+ in EGL
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Aspm+/+
AspmSA/SA
p=0.03
p=0.2
P7
P11


A.
% cC3+ cells in EGL at P7
p=0.01
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Aspm f/f
Aspm f/f p53 f/f
B.
% pH2AX+ cells in EGL at P7
p=0.6
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Aspm f/f
Aspm f/f p53 f/f
C.
% PH3+ cells in EGL at P7
p=0.9
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Aspm f/f
Aspm f/f p53 f/f
D.
cerebellum weight in μg at P30
p=0.0002
60
50
40
30
20
10
0
Aspm f/f
Aspm f/f p53 f/f


A.
B.
Aspm intact
Math1-cre;SmoM2;Aspm+/+
P3
P13
C.
D.
Aspm deleted
Math1-cre;SmoM2;Aspmf/f
E. ratio of crossectional area of tumor: non-tumor cerebellum in P13 Math1-cre;SmoM2 mice
4
3.5
3
2.5
2
1.5
1
0.5
0
p=0.04
Aspm+/+
Aspmf/f
P13
F.
P3 hGFAP-cre;SmoM2 tumors
Aspm: f/+ f/+ f/f f/f f/f
pH2AX
b-Actin